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The Contents of Case 09849967

Qnum	Query	DB Name	Thesaurus	Operator	Plural
Q1	hnrnp protein	USPT	ASSIGNEE	ADJ	YES
Q2	Q1 and splic$4	USPT	ASSIGNEE	ADJ	YES
Q3	Q2 and nucleotide binding	USPT	ASSIGNEE	ADJ	YES

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L2: Entry 5 of 27 File: USPT Sep 25, 2001

DOCUMENT-IDENTIFIER: US 6294332 B1
TITLE: Composition and methods for modulating the length of telomeres

Brief Summary Text (14):
Heterogeneous nuclear ribonucleoprotein particles (hnRNP) proteins are abundant proteins mammalian cells, of which the A to U members have been best characterized due to their RNA binding properties (Dreyfuss et al., 1993, Ann. Rev. Biochem. 62:289). There are over 20 such hnRNP proteins in human cells. The best characterized hnRNP protein so far is the hnRNP A1 protein which has been shown to be involved in alternative RNA splicing (Mayeda et al., 1992, Cell 68:365-375; Yang et al., Proc. Natl. Acad. Sci. USA. 91:6924-6928). Indeed, the hnRNP A1 protein has high affinity for RNA in vitro (Bird and Dreyfuss, 1994, EMBO J. 13:1197-1204). UP1 is a proteolytic product derived from hnRNP A1 (Riva et al., 1986, EMBO J. 5:2267-2273). UPI has no activity in alternative splicing in vitro (Mayeda et al., 1994, EMBO J. 13:5483-5495). In vitro experiments have shown that hnRNP A1 binds to oligonucleotides containing vertebrate 3' splice site sequences (Buvoli et al., 1990, Nucl. Acids Res. 18:6595). The DNA version of 3' splice site sequences share some similarity with vertebrate telomeric repeats (Ishikawa et al., 1993, Molec. Cell. Biol. 13:4301-4310). In vitro data concerning hnRNP A1 (UP1) can be summarized as follows: (1) A1 binds to DNA oligonucleotides carrying 3' splice site sequences which contain TTAGGT(Buvoli et al., 1990, Nucl. Acids Res. 18:6595); (2) UP1 is part of complexes assembled on oligonucleotides carrying telomeric repeats (TTAGGG)n (Ishikawa et al., 1993, Molec. Cell. Biol. 13:4301-4310), indicating that A1 and/or UP1 "could perhaps" bind to chromosome telomeres. However, this in vitro result is not yet correlated with in vivo data and cannot demonstrate th direct interaction of A1 and/or UP1 with telomeric repeats in the complex formed. Moreover, the oligonucleotides used in the expement which carry telomeric repeats also resemble the 3' splice site oligo used by Buvoli et al., 1990, Nucl. Acids Res. 18:6595. This could mean that (UP1) binds to 3' splice site sequences and that the telomeric sequence happens to resemble a 3' splice site (DNA version), and (3) Bird and Dreyfuss, 1994, EMBO J. 13:1197-1204 show that A1 binds to RNA and that the optimal sequence recognized by A1 resembles a 3' splice site and a 5' splice site. Although the optimal recognition sequence also resembles a telomeric repeat, the hypothesis that A1 might bind to telomeres appears to have been discarded. Models in which A1 binds to its preferred sequence in the context of an RNA molecule to modulate splicing, transport and possibly translation appear to be favoured (Bird and Dreyfuss, 1994, EMBO J. 13:1197-1204).

Brief Summary Text (17):
Proteins that can bind to single-stranded telomeric repeats in vitro include protein .alpha. and .beta. of Oxytrichia and Stylonychia, a of Euplotes, MF3 of chicken and XTEF of Xenopus. There has been no demonstration that the vertebrate proteins MF3 and XTEF bind to telomeres in vivo and no suggestion that their expression influences the size of telomeres. The yeast NSR1, GBP2, cdc13/EST4 and EST1 proteins were shown to bind to single-stranded yeast telomeric DNA (Lin and Zakian, 1994, Nucl. Acids Res. 22:4906; Nugent et al., 1996, Science 274:249; Virta-Pearlman et al., 1996, Genes Dev. 10:3094). While NSR1 and GBP2 do not affect telomere length in vivo, mutant strains engineered not to exress cdc13p or to express mutated forms of EST1 undergo telomere attrition despite having wild-type amounts of telomerase activity (Nugent et al., 1996, Science 274:249; Virta-Pearlman et al., 1996, Genes Dev. 10:3094). A limited number of mammalian proteins, including hnRNP proteins, have been reported to associate with oligonucleotides carrying telomeric repeats (Ishikawa et al., 1993, Molec. Cell. Biol. 13:4301-4310; McKay and Cooke, 1992, Nucl. Acids Res. 20:6461-6464; de Lange, 1996, Seminars in Cell & Dev. Biol. 7:23-29; Sang et al., 1997, J. Biol. Chem. 272:4474-4482). Whether any of these proteins bind to single-stranded telomeric repeats in cells and affect telomere length in vivo has yet to be documented. In any event the observation that a protein binds to telomeric sequences in vitro cannot be considered predictive of a role on the size of telomeres in vivo.

Brief Summary Text (23):
The applicant has determined that cells that do not contain detectable amounts of hnRNP A1 have drastically shortened telomeric repeat length as compared to cells that contain normal levels of hnRNP A1. The applicant has also determined that restoring hnRNP A1 expression in hnRNP A1-deficient cells promotes a gradual increase in the length of telomeres. Moreover, the applicant has also determined that UP1, a proteolytic product of hnRNP A1 which has lost its activity in alternative splicing (Mayeda et al. 1994, EMBO J. 13:5483), also promotes a gradual increase in the length of telomeres when expressed in hnRNP A1-deficient cells. Thus, the applicant is the first to provide data ta hnRNP A1 and its derivative, UP1, influence the length of

telomeres, thereby ruling out an indirect role through alternative splicing but rather giving rise to a factor involved in telomere length biogenesis or maintenance. Prior to applicant's experiments reported herein, there was no consensus by those in the art that one could predict that such experiments would provide the data observed by applicant or that such manipulations would have therapeutic utility.

Drawing Description Text (3):
FIG. 1 shows that hnRNP A1 expression Set telomere length (A) Western analysis of hnRNP A1 expression in CB3 cells and derivatives stably restored for A1 expression. Total proteins (5 .mu.g) from various mouse erythroleukemic cells were separated by SDS/PAGE. Following transfer onto nitrocellulose, the monocional antibody 9H10 (Bird et al., 1994, EMBO J. 13:1197, kindly provided by G. Dreyfuss) was used to detect hnRNP A1 proteins by ECL (AmershamLife Sciences). DP27-17 (lane 1) and CB7 (lane 5) cells are mouse erythroleukaemic cell lines (Shibuya et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3721; Ben-David et al., 1991, Genes Dev. 5:908) that express normal levels of hnRNP A1 proteins. CB3 (lane 2) cells are deficient in hnRNP A1 expression (Ben-David et al., 1992, Mol. Cell. Biol. 12:4449; Yang et al., 1994, Proc. Natl. Acad. Sc. U.S.A 91:6924). CB3-A1 (lane 3) cells are CB3 cells that have been stably restored for hnRNP A1 expression, by infection with a recombinant retrovirus. Retroviral vectors were constructed by inserting the mouse A1 cDNA (Ben-David et al., 1992, Mol. Cell. Biol. 12:4449) into the EcoRl site of pMSLV, a retroviral vector containing the neomycin resistance gene driven by the pgk promotor (Hawley et al., 1992, J. Exp. Med. 176:1149).Recombinant retroviral vectors were transfected into GP+-A86 cells (Hawey et al., 1992, J. Exp. Med. 176:1149).Following selection in the presence of G418, viral stocks were harvested and their titers determined. CB3 cells were then infected with each viral stock and pools of infected cells were selected in medium containing G418 (800 .mu.g/ml for the first week and 400 .mu.g/ml thereafter).Cells were seeded twice weekly at a 1:20 split ratio. CB3-Ala (lane 4) cells were produced by infection of CB3 cells with a retrovirus carrying the A1 cDNA in reverse orientation. The position of molecular weight marker is indicated at left; the respective cell lines from which the protein extract were prepared are indicated on the top of each lane; the positions of the A1 protein and its spliced isoform A1b are shown at right (B, C) Genomic DNA was extracted from mouse erythroleukemic cells, digested with Hinfl and Rsal. For each lane, 5 .mu.g of digested DNA was loaded onto the gel. The molecular size standard DNA was a mixture of .sup.32 P-end-labelled ladder DNA (GIBCO) and unlabelied Hindlll-digested lambda DNA. To confirm the identity of the telomeric signals detected in CB7 and CB3 cells, genomic DNA was digested with the Bal31 exonuclease prior to treatment with restriction endonucleases. This treatment abolished the telomeric signals while preserving the detection of restriction fragments containing internal sequences repeats in the mouse genome (data not shown). The restricted DNA was separated on a 0.5% agarose gel and hybridized to the mammalian telomeric probe .sup.32 P-(TCCCTAA).sub.3. In panel C, the DNA from mock-infected CB83 cells or CB3 cells infected with the A1 or A1.alpha. viruses was analysed after 46 cell passages. The position and size (in kb) of molecular weight markers (M) are indicated (lanes 1 in panels B and C).

Detailed Description Text (4):
Given that hnRNP A1 modulates alternative RNA Splicing (Yang et al., 1994, Proc. Natl. Acad. Sci. 91:6924; Mayeda et al., 1992, Cell 68:365; Caeres et al., 1994, Science 265:1706), it is possible that hnRNP A1 influences splice site selection in an alternatively spliced pre-mRNA whose product affects telomere length. To address whether the effect of A1 on telomeres was dependent of its activity as an alternative splicing factor, we tested the effect of expressing UP1 in CB3 cells. UP1 is a proteolytic fragment of hnRNP A1 initially identified as a single-standed DNA binding protein (Riva et al., 1986, EMBO J. 5:2267; Herrick et al., 1976, J. Biol. Chem. 251:2124-2133; Cobianchi et al., 1988, J. Biol. Chem. 263:1063; Casas-Finet et al., 1993, J. Mol. Biol. 229:873). UP1 completely lacks activity in alternative splicing (Mayeda et al., 1994, EMBO J. 13:5483). A UP1 recombinant retrovirus was produced and used to infect CB3 cells. Briefly, the UP1 cDNA fragment was produced by PCR amplification of a mouse A1 cDNA clone using oligonucleotides A1 M [5'-AATTCTTTTGCTCGACGCTGCCGAG-3'; SEQ ID NO:4 the underlined sequence maps at the extreme 5' end of the murine A1 cDNA (Ben-David et al., 1992, Mol. Cell. Biol. 12:4449)], and A1-1647 [5-AATTCTGTCAGCGACCTCTCTGACTGGATGA-3'SEQ ID NO:5; the underlined sequene is complementary to the region encoding the carboxy-terminal domain of UP1]. PCR amplification leads to the inclusion of a stop codon immediately following the codon specifying the last amino acid in UP1. The 661 nt UP1 was subcioned, sequenced and inserted as an EcoRI fragment into the EcoRI site of pMSCV. Viral stocks were produced and used to infect CB3 cells as described earlier. Because the epitope recognized by the anti-A1 antibody is absent from the UP1 protein, a RT-PCR assay was used to monitor UP1 expression (FIG. 2A). Genomic DNA was isolated at regular intervals after infection and analyzed for changes in the length of TRFs. By comparison to a parallel infection of the same CB3 cell culture with the A1.alpha. virus (FIG. 2B, lanes 2-5), infection with the UP1 virus led to a gradual increase in the size of TRFs, the bulk of the fragments migrating in the 8-12 kb range after 57 cell passages (FIG. 2B, lanes 6-9). Further passages showed that the lengths of the telomeres kept on increasing. Indeed, at 86 passages the lengths of telomeres in CB3-UP1 cells was shown to comigrate with that of CB7 (data not shown). An increase in TRF size and hybridization intensity such as observed when hnRNP A1 (FIG. 1C, lane 3) or UP1 (FIG. 2B, lane 9) was expressed, was never observed in mock-infected CB3 cells or CB3 cells infected with the A1.alpha. virus. Thus, the increase in TRF size is specific to A1 and UP1 expression in CB3 cells and is not due to clonal variation.

Detailed Description Text (7):
The gradual changes in the telomeric repeat tracts following restoration of hnRNP A1 or UP1 expression in CB3 cells are reminiscent of a similar phenotypic lag associated with yeast telomere tract changes caused by Rap 1p overexpression (Conrad et

al., 1990, Cell 63:739), by mutations in the EST1 and EST4/CDC13 genes (Nugent et al., 1996, Science 272:249; Lin et al., 1996, Proc. NaU. Acad. Sci. U.S.A 93:13760; Ludblad et al., 1989, Cell 57:633) or by certain RAP1 mutant alleles (Lustig et al., 1990, Science 250:549; Kyrion et al., 1992, Mol. Cell. Biol. 12:5159). HnRNP A1 does not share significant similarity with these and other yeast proteins that bind to single-stranded telomeric repeats or affect telomers length (Nugent et al., 1996, Science 272:249; Virta-Pearlman et al., 1996, Genes Dev. 10:3094;Greenwell et al., 1995, Cell 82:823; Morrow et al., 1995, Cell 82:831; Runge et al., 1996, Mol. Cell. Biol. 16:3094), nor with Tetrahymena (Collins et al., 1995, Cell 81:677) or Oxytricha (Hicke et al., 1990, Proc. Natl. Acad. Sci. U.S.A 87:1481; Gray et al., 1991, Cell 67:807) proteins that are either components of the telomerase or associate with telomeres in vivo. HnRNP A1 is also quite distinct from the human TRF protein which associates with double-stranded telomeric repeats (Zhong et al., 1992, Mol. Cell. Biol. 12:4834; Hanish et al., 1994, Proc. Natl. Acad. Sci. U.S.A 91:8861; Chong et al., 1995, Science 270:1663). Whereas members of the family of hnRNP proteins other than hnRNP A1 can associate with telomeric repeats in vitro (Ishikawa et al., 1993, Molec. Cell. Biol. 13.4301; McKay et al., 1992, Nucleic Acids Res. 20:6461), their presence in CB3 cells did not appear sufficient to prevent telomere shortening. Notably, the deficiency in hnRNP A1 was not associated with an absence or severely reduced telomerase activity, nor did restoring hnRNP A1 or UP1 expression affect telomerase activity in vitro. This result suggests that hnRNP A1/UP1 is not itself an obligatory part of the active telomerase complex nor is it required, at least in vitro, for substrate presentation to the telomerase. Our results raise the possibility that the proteolytic processing of A1 into UP1 may be part of the normal pathway that modulates oFe fi dAlin telomere biogenesis The binding of A1 or UP1 to single-band DNA carrying vertebrate TAGGGT repeats suggests possible mechanisms by which A1/UP1 can promote telomere elongation. HnRNP A1/UP1 may protect the exposed single-stranded tails from nudeolytic degradation. Because UP1 stimulates DNA polymerase a (Riva et al., 1986, EMBO J. 5:2267), UP1 binding to single-st telomeric DNA may also facilitate C-strand synthesis after telomerase elongation.

Detailed Description Text (65):
Based on the above results, generating transgenic mice exressing UP1 is potentiated. The choice of UP1 is preferred since this form does not intedere with alternative splicing, at least in vitro (Mayeda et al., 1994, EMBO J. 13:5493-95). Moreover, because hnRNP A1 is involved in at least two distinct molecular processes, the effects of aftering A1 expression in whole animals are unpredictable. Changes in the exprssion of A1 may be lethal or cause multiple defects due to one or several of te biological functions of A1. Transgenic animals will be generated by programming UP1 expression through different promotors including the MMTV promotor which allows to target tissue-specific expression in mammnary glands. A constitutive promotor (.beta.-actin) will be used to drive UP1 expression in all tissues. An increase in the tissue-specific or general expression of UP1 should promote the formation of abnormally long and stable telomeres. Telomere elongation may be associated with a greater protection against cancer by keeping in check genomic instability. Alternatively, it may offer a selective growing advantage on cells that express oncogenes or mutated anti-oncogenes. The consequence of alterations in telomere structure on embryonic development (viability and incidence of gross abnormalities), genomic instability (gross chromosomal abnormalities determined by karyotype analysis and rate of spontaneous mutation in a reporter gene), tumor occurrence and susceptibility to mutagenic or tumorigenic agents (e.g. radiation), growth rate and frequency of spontaneous immortalization of primary fibroblasts will be followed. The effect of UP1 expression on tumor development will be further verified by performing crosses between MMTV/UP1 transgenic animals and syngenic MMTV/neu transgenics (Jackson Labs., Maine), a strain that displays a high incidence of mammary tumors.

Other Reference Publication (2):
Pandolfo et al., Single stranded DNA binding proteins derived from hnRNP proteins by proteolysis in mammalian cells. vol. 13, No. 18, pp. 6576-6590. Sep. 1985.*

Other Reference Publication (3):
Pandolfo et al., Single stranded DNA binding proteins derive from hnRNP proteins by proteolysis in mammalian cells, 1985, Nucleic Acids Research, 13: 6576-6590.

Other Reference Publication (5):
Buvoli et al., Recombinant hnRNP protein A1 and its N-terminal domain show preferential affinity for oligodeoxynucleotides homologous to intron/exon acceptor sites, 1990, Nucleic Acids Research, 18: 6595-6600.

Other Reference Publication (9):
Dreyfuss et al., hnRNP Proteins and the Biogenesis of mRNA, 1993, Annu. Rev. Biochem, 62:289-321.

Other Reference Publication (10):
Ishikawa et al., Nuclear Proteins That Bind the Pre-mRNA 3' Splice Site Sequence r(UUAG/G) and the Human Telomeric DNA Sequence d(TTAGGG)n, 1993, Molecular and Cellular Biology, USA, 4301-4310.

Other Reference Publication (11):
Burd et al., RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing, 1994, The Embo Journal, England, 13: 1197-1204.

Other Reference Publication (15):
Yang et al., The A1 and A1B proteins of heterogenous nuclear ribonucleoparticles modulate 5' splice site selection in vivo, 1994, Proc. Natl. Sci, USA, 91: 6924-6928.

1: Science 1994 Sep 16;265(5179):1706-9

Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors.

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The opposing effects of SF2/ASF and heterogeneous nuclear ribonucleoprotein (hnRNP) A1 influence alternative splicing in vitro. SF2/ASF or hnRNP A1 complementary DNAs were transiently overexpressed in HeLa cells, and the effect on alternative splicing of several cotransfected reporter genes was measured. Increased expression of SF2/ASF activated proximal 5' splice sites, promoted inclusion of a neuron-specific exon, and prevented abnormal exon skipping. Increased expression of hnRNP A1 activated distal 5' splice sites. Therefore, variations in the intracellular levels of antagonistic splicing factors influence different modes of alternative splicing in vivo and may be a natural mechanism for tissue-specific or developmental regulation of gene expression.

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